



King's Research Portal

DOI:

[10.1093/hmg/ddt313](https://doi.org/10.1093/hmg/ddt313)

Document Version

Publisher's PDF, also known as Version of record

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Vagnoni, A., Glennon, E., Perkinton, M., Gray, E., Noble, W., & Miller, C. (2013). Loss of c-Jun N-terminal kinase-interacting protein-1 does not affect axonal transport of the amyloid precursor protein or A production. *Human Molecular Genetics*, 22(22), 4646-4652. [N/A]. <https://doi.org/10.1093/hmg/ddt313>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Loss of c-Jun N-terminal kinase-interacting protein-1 does not affect axonal transport of the amyloid precursor protein or A β production

Alessio Vagnoni^{†,‡}, Elizabeth B.C. Glennon[†], Michael S. Perkinson[§], Emma H. Gray[¶], Wendy Noble and Christopher C.J. Miller*

Departments of Neuroscience and Clinical Neurosciences, Institute of Psychiatry, King's College London, London, UK

Received May 29, 2013; Revised and Accepted June 27, 2013

Disruption to axonal transport is an early pathological feature in Alzheimer's disease. The amyloid precursor protein (APP) is a key axonal transport cargo in Alzheimer's disease since perturbation of its transport increases APP processing and production of amyloid- β peptide (A β) that is deposited in the brains of Alzheimer's disease patients. APP is transported anterogradely through axons on kinesin-1 motors. One favoured route for attachment of APP to kinesin-1 involves the scaffolding protein c-Jun N-terminal kinase-interacting protein-1 (JIP1), which has been shown to bind both APP and kinesin-1 light chain (KLC). However, direct experimental evidence to support a role of JIP1 in APP transport is lacking. Notably, the effect of loss of JIP1 on movement of APP through axons of living neurons, and the impact of such loss on APP processing and A β production has not been reported. To address these issues, we monitored how siRNA mediated loss of JIP1 influenced transport of enhanced green fluorescent protein (EGFP)-tagged APP through axons and production of endogenous A β in living neurons. Surprisingly, we found that knockdown of JIP1 did not affect either APP transport or A β production. These results have important implications for our understanding of APP trafficking in Alzheimer's disease.

INTRODUCTION

The correct transport of protein and organelle cargoes through axons (axonal transport) is an essential requirement for proper functioning of neurons. Since most proteins are synthesized in neuronal cell bodies, axonal and synaptic cargoes need to be transported anterogradely through axons to their final destinations, whereas trophic factors and other cargoes are transported retrogradely from the synapse to the cell body. Indeed, perturbation of axonal transport is now known to contribute to the pathogenic process in some neurodegenerative diseases (see for reviews (1–4)).

Alzheimer's disease is the most common form of dementia, but the molecular mechanisms that cause disease are not fully understood. However, altered metabolism of amyloid precursor protein (APP) leading to changes in production of amyloid- β peptide (A β) is believed to be central to the disease process.

A β is an approximately 40–42 amino acid peptide that is derived from APP by proteolytic processing and which is deposited in the brains of Alzheimer's disease patients within amyloid plaques. Generation of A β involves processing by β -site APP cleaving enzyme-1 (BACE1) and γ -secretase that cleave APP at the N- and C-termini, respectively, of the A β sequence. In addition, APP is processed by α - and γ -secretase but since α -secretase cleaves within the A β sequence, this route precludes A β production (see for review (5)). A large body of evidence suggests that at least some forms of A β have pathogenic properties (see for reviews (6,7)). As such, understanding the mechanisms that control APP processing and A β production has major relevance to Alzheimer's disease research.

One route for altering APP processing involves changes to its transport through axons. Thus, experimental disruption of anterograde axonal transport of APP increases production of A β

*To whom correspondence should be addressed at: Department of Neuroscience P037, Institute of Psychiatry, De Crespigny Park, Denmark Hill. London SE5 8AF. UK. Tel: +44 207 8480393; Email: chris.miller@kcl.ac.uk

[†]These two authors contributed equally to this study.

[‡]Present Address: MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge CB2 0QH UK.

[§]Present Address: AstraZeneca Neuroscience iMED, MedImmune Limited, Aaron Klug Building, Granta Park, Cambridge, CB21 6GH UK.

[¶]Present Address: MS Society, 372 Edgware Road, London NW2 6ND UK.

(8–10). Moreover, since A β itself disrupts axonal transport (11–17), one suggestion is that damage to axonal transport promotes a toxic cycle of events. In this scenario, disruption to APP transport increases A β production which, in turn, induces more damage to axonal transport to further elevate A β production (8). As such, APP represents a key axonal transport cargo in Alzheimer's disease.

APP is transported anterogradely through axons on kinesin-1 molecular motors (8,18–20). Most functional kinesin-1 is a heterotetramer of two kinesin-1 motor proteins and two kinesin-1 light chains (KLCs). Kinesin-1 contains the motor domain, whereas the KLC is mainly involved in binding of cargoes (21). KLC is required for APP transport (8,22), but the mechanism by which APP attaches to KLC is not clear.

In one model, the intracellular C-terminus of APP binds directly to KLC to facilitate transport, but some studies have queried this finding (23,24). Recently, calyntenin-1 (also known as alcadin- α) has been shown to be involved in APP transport (9,25). Calyntenin-1 is a type-1 membrane-spanning protein that binds directly to KLC via its intracellular C-terminus, so as to mediate post-Golgi transport of a subset of vesicles through axons (26–30). In this model, APP does not directly attach to KLC or kinesin-1 motors but rather is loaded onto calyntenin-1 containing vesicle carriers for transport (9,10,25). Finally, APP may attach to KLC via intermediary scaffolding proteins such as c-Jun N-terminal kinase-interacting protein-1 (JIP1) or PAT1 (27,31–36). JIP1 is the favoured scaffold since it binds to both APP and KLC in biochemical assays and a proportion of APP and JIP1 colocalize in axons (27,32,36–41). Moreover, axonal transport of APP involving JIP1 has been implicated in APP processing and A β production (27,41).

However, much of the data supporting a role of JIP1 in axonal transport and processing of APP involve analyses of fixed cells or cell lines overexpressing JIP1 and/or APP (27,32,35,36). Notably, the effect of loss of JIP1 on axonal transport of APP in living neurons and how such loss affects production of endogenous A β have not been reported. Clearly, a proper analysis of the role of JIP1 in APP transport requires dynamic studies of APP movement. Here, we address these issues by analyses of neurons in which JIP1 expression is downregulated with siRNAs.

RESULTS

siRNA loss of JIP1 does not influence A β production, APP processing at the α - or β -secretase sites or phosphorylation of APP at threonine-668

We first tested the role of JIP1 in the production of endogenous A β in rat cortical neurons. To do so, we utilized siRNAs to knock down JIP1. In the neurons, JIP1 migrated as two major species which is consistent with previous reports and is probably the result of differential phosphorylation since JIP1 is a known phosphoprotein (42,43). Two different siRNAs and a mixture of both siRNAs all reduced JIP1 to undetectable levels (Fig. 1A). However, A β ELISAs revealed that this reduction in JIP1 did not affect secretion of either A β (1–40) or A β (1–42) into the media (Fig. 1B and C). For comparison and as controls, we also treated the neurons with siRNAs for calyntenin-1 and with the γ -secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-1-alanyl]-*S*-phenylglycine-*t*-butyl ester (DAPT). Loss of

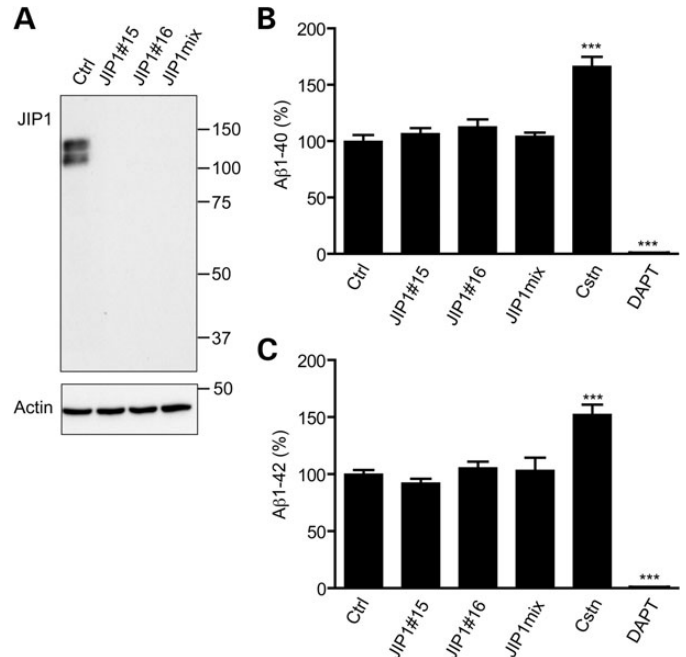


Figure 1. siRNA knockdown of JIP1 does not affect the production of A β . (A) siRNA knockdown of JIP1 in rat cortical neurons. Neurons were treated with control (Ctrl) siRNA or with two different JIP1 (JIP1#15 and JIP1#16) or a mixture of both JIP1 siRNAs (JIP1mix). Samples were then probed on immunoblots for JIP1 or actin as a loading control. Molecular mass markers are shown. (B) ELISAs showing relative A β (1–40) and A β (1–42) levels (expressed as a percentage of control values) in conditioned media from neurons treated with control siRNA, JIP1#15, JIP1#16, JIP1mix or calyntenin-1 siRNAs. Also shown are neurons treated with DAPT. Statistical significance was determined by one-way ANOVA with a Bonferroni *post hoc* test. *N* = 6 error bars are SEM; ****P* < 0.001.

calyntenin-1 has been shown to disrupt axonal transport of APP to increase A β production, whereas DAPT inhibits γ -secretase cleavage of APP to decrease A β production (9,10,44). In agreement with these findings, siRNA knockdown of calyntenin-1 using a pool of siRNAs previously shown to reduce calyntenin-1 to levels <5% of that seen in control neurons (9) increased whereas DAPT decreased production of A β (1–40) and A β (1–42) (Fig. 1B and C).

We next enquired whether siRNA knockdown of JIP1 influenced APP expression or its phosphorylation on threonine-668. JIP1 has been reported to preferentially bind to APP phosphorylated on threonine-668 (32). Since the two individual and mix of both JIP1 siRNAs all reduced JIP1 levels to the same extent (Fig. 1A), we utilized the siRNA mix for these experiments. siRNA loss of JIP1 did not alter total APP levels or the levels of APP phosphorylated on threonine-668 in the neurons (Fig. 2A).

We also enquired whether siRNA loss of JIP1 influenced APP processing at the α -secretase and BACE1 sites. Processing of APP by α -secretase and BACE1 generates N-terminal secreted APP fragments (sAPP α and sAPP β), which can be detected in conditioned media from cells using antibodies that specifically recognize each sAPP fragment. Immunoblotting of conditioned media from control or JIP1 siRNA knockdown neurons with antibody 22C11 (that detects total sAPP) and antibodies that detect sAPP α and sAPP β revealed that loss of JIP1 did not influence the levels of total sAPP, sAPP α or sAPP β (Fig. 2A). These

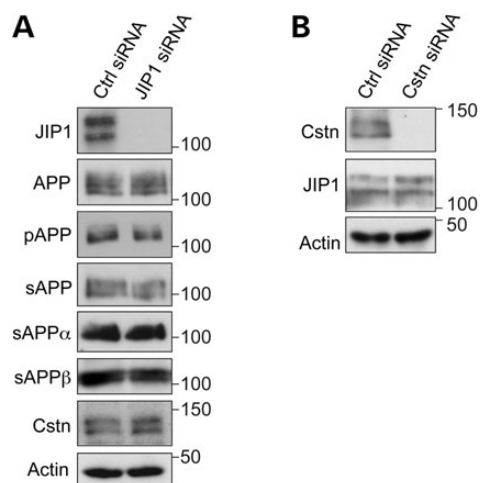


Figure 2. (A) siRNA knockdown of JIP1 does not affect APP processing or calyntenin-1 levels in rat cortical neurons. Neurons were treated with control (Ctrl) or JIP1 mix siRNAs (JIP1) and the samples probed on immunoblots for full-length APP (APP), APP phosphorylated on threonine-668 (pAPP), total sAPP, sAPP α and sAPP β in conditioned media, and calyntenin-1 (Cstn); actin is shown as a loading control. No significant changes in the levels of any of these proteins or phosphorylation of APP on threonine-668 were detected between control or JIP1-siRNA treated neurons (Student's *t*-test $n = 3$). (B) siRNA knockdown of calyntenin-1 does not affect JIP1 protein levels. Neurons were treated with control (Ctrl) or calyntenin-1 (Cstn) siRNAs and the samples probed on immunoblots for JIP1 and actin as a loading control. No significant changes in the levels of JIP1 were detected between control or calyntenin-1 siRNA-treated neurons (Student's *t*-test $n = 3$).

findings are consistent with the A β assays, which also suggest that JIP1 knockdown does not affect APP processing.

Finally, we enquired whether loss of JIP1 influenced calyntenin-1 levels in the neurons since APP is transported through axons by kinesin-1 on calyntenin-1 carriers and loss of calyntenin-1 affects APP processing (9,10,25). However, loss of JIP1 had no discernible effect on calyntenin-1 levels (Fig. 2A). Thus, loss of JIP1 in neurons does not influence endogenous APP levels, phosphorylation of endogenous APP at threonine-668, processing of endogenous APP at the α -secretase or BACE1 sites or production of endogenous A β .

Since loss of calyntenin-1 affects APP processing, A β production and axonal transport of APP (9,10,25) (and see Fig. 1), we also monitored JIP1 levels in neurons treated with calyntenin-1 siRNAs. However, we detected no difference in JIP1 levels between control and calyntenin-1 siRNA knockdown neurons (Fig. 2B).

siRNA loss of JIP1 does not noticeably influence axonal transport of APP

To examine the role of JIP1 in the axonal transport of APP, we quantified movement of enhanced green fluorescent protein (EGFP)-tagged APP (APP-EGFP) through axons of transfected living rat cortical neurons following siRNA knockdown of JIP1 using time-lapse microscopy. To avoid any possible artifacts produced by high levels of APP-EGFP expression, we chose for study, cells expressing low levels of APP-EGFP as judged by brightness of the EGFP signal. This approach is line with our previous studies (9,29,45). Kymographs were generated

from the time-lapse movies, and these were used to calculate the velocities and flux rates of APP-EGFP movement in both anterograde and retrograde directions. Flux rates were determined by counting the numbers of APP-EGFP particles that crossed a defined line within the mid-section of axons as previously described by us and others (8,9,46). In control siRNA treated neurons, APP-EGFP moved through axons in a predominantly anterograde direction at fast speeds of between $\sim 1-9 \mu\text{m/s}$ and $1-5 \mu\text{m/s}$ in the anterograde and retrograde directions, respectively. These velocities and the bias towards anterograde movement are similar to those described previously for movement of fluorescent protein-tagged APP in neurons (8,9,18,19,46-48). siRNA knockdown of JIP1 did not significantly alter the velocities of APP-EGFP movement in either anterograde or retrograde directions, the bias towards anterograde movement, or the flux rates of anterograde or retrograde movements (Fig. 3) (and see Supplementary data). Thus, loss of JIP1 has no noticeable effect on axonal transport of APP through axons of living rat cortical neurons.

The levels of JIP1 are not altered in Alzheimer's disease brains

To begin to determine whether JIP1 is altered in Alzheimer's disease, we monitored the levels of JIP1 protein in control and Braak stage V-VI Alzheimer's cortical brain samples (see Table 1, for details of patient samples). The age at death and post-mortem delay was not significantly different between the control and Alzheimer's disease samples. However, we found no differences in the levels of JIP1 between the two sample sets (Fig. 4).

DISCUSSION

APP is transported anterogradely through axons by kinesin-1 motors (8,18-20), but the mechanisms that mediate attachment of APP to kinesin-1 are not properly understood. JIP1 is a ligand for KLC and via its PTB domain also binds to the C-terminus of APP (27,32,36-41). As such, JIP1 has been favoured as a scaffold to connect APP to KLC/kinesin-1 for anterograde axonal transport (27,32,35,36). Here, we directly tested the role of JIP1 in axonal transport of APP by quantifying APP-EGFP movement in JIP1 siRNA knockdown neurons. Despite highly efficient knockdown of JIP1, we detected no changes in APP movement in either anterograde or retrograde directions following loss of JIP1.

Disruption to axonal transport of APP influences its processing and production of A β (8-10). Others have shown that overexpression of JIP1 decreases A β production but these studies were conducted in non-neuronal or neuroblastoma cells overexpressing APP (27,41). Our analyses revealed no effect of loss of JIP1 on endogenous A β production in bona fide neurons, and these findings are consistent with the absence of an effect of JIP1 loss on axonal transport of APP in the same neurons. Of course, it is possible that JIP1 facilitates axonal transport of a proportion of APP on kinesin-1 motors but that in the JIP1 siRNA knockdown neurons, this effect is not noticeable due to switching of APP transport to other kinesin-1 carriers, such as those containing calyntenin-1 so as to rescue any phenotype. Calyntenin-1 has recently been shown to mediate transport of

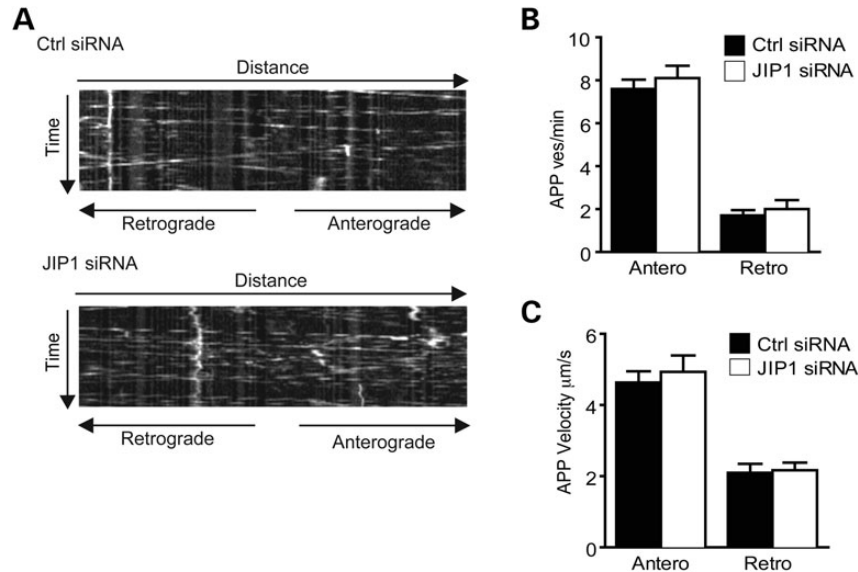


Figure 3. siRNA knockdown of JIP1 does not effect axonal transport of APP-EGFP. (A) Representative kymographs showing axonal transport of APP-EGFP in control and JIP1 siRNA-treated neurons. (B) and (C) show bar charts of APP-EGFP vesicle movement in control (Ctrl) and JIP1 siRNA treated neurons. (B) shows APP-EGFP flux rates (as APP vesicles/minute) and (C) shows APP-EGFP velocities in both anterograde and retrograde directions. Statistical significance was determined by Student's *t*-test. *n* = 26 control and 49 JIP1 siRNA treated cells. Error bars are SEM.

Table 1. Data for human post-mortem samples showing age at death, post-mortem delay (PMD) and pathological diagnosis

Case	Age (years)	Sex	PMD	Pathological Diagnosis
A141/07	80	M	41	Alzheimer's disease Braak VI
A065/04	91	F	29	Alzheimer's disease Braak VI
A094/04	88	M	46	Alzheimer's disease Braak VI
A059/07	92	F	42	Alzheimer's disease Braak VI
A331/07	80	F	13	Alzheimer's disease Braak V
A191/07	69	F	16	Alzheimer's disease Braak VI
A210/05	84	F	<24	Alzheimer's disease Braak V
A167/05	81	F	<72	Alzheimer's disease Braak VI
A187/07	82	F	69	Alzheimer's disease Braak V
A168/05	84	F	36	Alzheimer's disease Braak V/VI
A247/05	90	F	23	Alzheimer's disease Braak V/VI
A350/09	98	F	3.5	Alzheimer's disease Braak V
A065/02	82	M	80	Alzheimer's disease Braak V/VI
A318/09	72	M	5	Alzheimer's disease Braak VI
A181/09	89	F	15	Alzheimer's disease Braak V
A240/06	97	F	12	Alzheimer's disease Braak V
A013/03	89	M	19	Alzheimer's disease Braak V
A122/04	86	M	26	Alzheimer's disease Braak V
A332/07	87	F	48	Alzheimer's disease Braak VI
A140/07	81	F	17	Control
A153/06	92	F	17	Control
A134/00	86	M	6	Control
A358/08	55	F	12	Control
A192/09	80	M	21	Control
A063/10	90	F	50	Control
A239/03	78	M	10	Control
A265/08	79	M	47	Control
A040/07	82	F	13	Control
A124/04	59	M	50	Control

APP by kinesin-1 (9,10). Thus, while our results cannot formally eliminate any role of JIP1 in kinesin-1 transport of APP, the absence of any noticeable effect of loss of JIP1 on APP movement, APP processing or A β production together argue against

a major role for JIP1 in axonal transport of APP at least in the cortical neurons utilized here.

In contrast to an absence of effect of JIP1, siRNA loss of calyntenin-1 reduces axonal transport of APP to increase A β production (9,10) (and see Fig. 1). Calyntenin-1 is a type-1 membrane-spanning protein that attaches via its intracellular C-terminus to KLC (26–28). Calyntenin-1 also binds to the adaptor protein X11 β (also known as X11-like and munc-18-interacting protein-2) (49). There is evidence that X11 β functions as a coat protein to direct loading of cargoes into particular vesicle sub-types (50). X11 β also binds to the C-terminus of APP (51,52) and like calyntenin-1, modulating X11 β expression affects APP processing and A β production (53–55). Thus, X11 β may load APP onto calyntenin-1 containing vesicles for post Golgi transport by kinesin-1 through axons and disruption to this transport either via loss of calyntenin-1 or X11 β may promote amyloidogenic processing of APP. JIP1 has been reported to be mislocalised in Alzheimer's disease (56) but we detected no changes in the total levels of JIP1 in Alzheimer's disease brains. This contrasts with calyntenin-1 where both decreased protein levels and altered processing have been reported (9,57,58). Thus, defective axonal transport of APP in Alzheimer's disease may be more closely linked to defects in calyntenin-1 rather than JIP1 mediated cargo transport.

MATERIALS AND METHODS

Plasmids and siRNAs

APP-EGFP was as described (9,59). Verified non-targeting control and rat JIP1 siRNAs (#15 5'-CUGCUAUGCAAAA GAUCGC-3', #16 5'- CCAUGUUUUGGAGAUUACA-3') were purchased from Dharmacon (Accell range). Calyntenin-1 pool siRNAs were as described (9) and also obtained from Dharmacon.

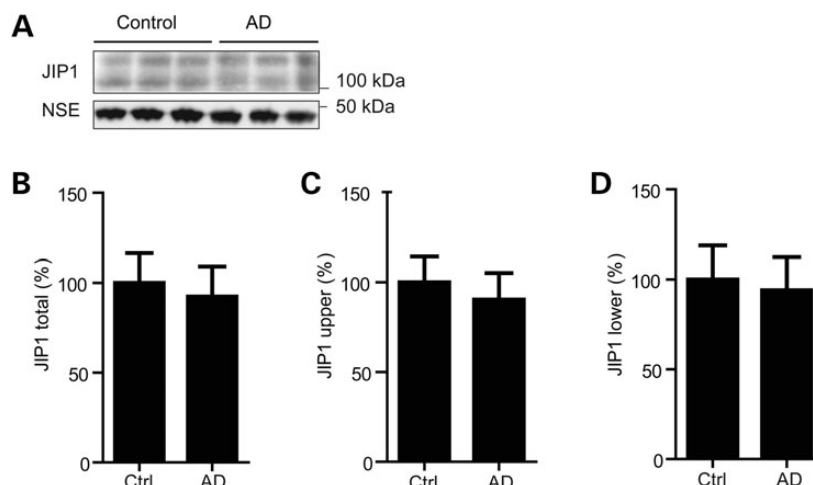


Figure 4. JIP1 levels are not altered in Alzheimer's disease brains. (A) Immunoblots of representative samples from control and Alzheimer's disease frontal cortex. Samples were probed for JIP1 and NSE. (B–D) Bar charts showing relative levels (%) of JIP1 in the two sample sets following normalization to NSE signals in each sample. Values shown are for total JIP1 (upper and lower species combined), upper JIP1 and lower JIP1 species. Data were tested for normality using a Kolmogorov–Smirnov normality test, and differences between groups determined by a Mann–Whitney test. $n = 10$ control and $n = 19$ Alzheimer's disease samples. Error bars are SEM.

Antibodies

Rabbit APP C-terminal and calsynenin-1 antibodies were as described (9,29,60,61). Mouse monoclonal antibodies to beta actin (clone AC-15) were from Abcam (Cambridge, UK) and a JIP-1 mouse monoclonal antibody (clone B-7) was from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-APP mouse clone 22C11 was from Merck Millipore (Billerica, USA); anti-sAPP α clone 2B3 and anti-sAPP β rabbit polyclonal antibodies were from IBL International (Hamburg, Germany), anti-phospho-threonine-668 APP rabbit polyclonal antibody was from Cell Signaling Technology (Danvers, USA). Anti NSE antibody was from Dako (Cambridge).

Cell culture and transfection

Cortical neurons were obtained from embryonic day 18 rat embryos and cultured in Neurobasal medium containing B27 supplement, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (Invitrogen). For siRNA studies, neurons at DIV4 were treated with 1 μ M of each siRNA (control, JIP1 or calsynenin-1) for 96 h and then analysed at DIV8. For transfection studies, neurons were cultured on poly-L-lysine-coated glass cover slips in 12-well plates. Neurons were transfected on a magnetic plate for 30 min using 1 μ g plasmid DNA and 2 μ l magnetic nanoparticles (NeuroMag, OZ Biosciences) as described by the manufacturer. Neurons were transfected on DIV7 and analysed on DIV8.

Measurement of A β production

To determine how loss of JIP1 affects production of endogenous A β (1–40) and A β (1–42), conditioned medium from rat cortical neurons at DIV4 cultured in 24-well plates (2.5×10^5 cells per well) was removed and replaced with 400 μ l fresh Neurobasal/B27 medium containing 1 μ M control, JIP1 or calsynenin-1 siRNAs. The neurons were then returned to the incubator for a

further 96 h and the conditioned media then assayed for A β (1–40) and A β (1–42). Some neurons were also treated with 2 μ M of the γ -secretase inhibitor DAPT. For A β assays, the conditioned media was removed and centrifuged at $100000 \times g$ for 30 min at 4°C to remove any insoluble material. Levels of A β (1–40) and A β (1–42) were then determined using quantitative mouse/rat A β ELISAs from Invitrogen (A β (1–40) ELISA KMB3481; A β (1–42) ELISA KMB3441) according to the manufacturer's instructions. Data were analysed using one-way ANOVA tests with Bonferroni *post hoc* test.

SDS–PAGE and immunoblotting

Cells were harvested for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) by washing with phosphate buffered saline pre-warmed at 37°C and scraping into SDS–PAGE sample buffer and immediately heating to 100°C. Total sAPP, sAPP α and sAPP β levels in conditioned media were likewise prepared for SDS–PAGE by addition of sample buffer at heating to 100°C.

Human brain samples were prepared as 20% homogenates and equal protein concentrations then prepared for SDS–PAGE by addition of SDS–PAGE sample buffer and heating to 100°C. Samples were separated on either 8 or 10% (w/v) acrylamide gels; separated proteins were then transferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a Transblot system (BioRad) and following blocking, probed with primary antibodies. Following washing, the blots were further probed with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Igs and developed using an enhanced chemiluminescence system (GE Healthcare).

Human tissues

All human tissue collection and processing were carried out under the regulations and licensing of the Human Tissue Authority and in accordance with the Human Tissue Act, 2004.

Time-lapse microscopy

Live microscopy of APP-EGFP was performed with an Axiovert S100 microscope (Zeiss) equipped with a Lambda LS Xenon-Arc light source (Sutter Instrument Company, Novato, USA), GFP filter set (Chroma Technology Corp., Rockingham, USA), 100 \times Plan-Apochromat 1.4 N.A. objective (Zeiss), Lambda 10–3 filter wheel (Sutter Instrument Co.) and a Photometrics Cascade-II 512B High Speed EMCCD camera (Photometrics, Tuscon, USA). Twenty-four hour post-transfection, the cells were transferred to a Ludin imaging chamber (Life Imaging Systems, Basel, Switzerland) mounted on the stage of the microscope. Neurons were maintained in a HEPES-buffered extracellular neuronal solution (composition in mM: NaCl, 140; KCl, 5; NaHCO₃, 5; MgCl₂·6H₂O, 1; CaCl₂, 1.2; Na₂HPO₄, 1.2; Glucose, 10; Hepes, 20; pH 7.4) at 37°C using an objective heater (IntraCell, Royston, UK) and 'The Box' Microscope Temperature Control System (Life Imaging Systems). Vesicle movements were recorded for 2 min with a 1 s time-lapse interval using MetaMorph software (Molecular Devices). Image analysis was performed with ImageJ. The flux rates for APP-EGFP transport in both anterograde and retrograde directions were determined essentially as described (8,9,46). This involved quantifying the numbers of APP-EGFP particles that crossed a defined line in mid-axons per minute. APP-EGFP velocities were determined from kymographs, essentially as described for other cargoes (62). Statistical significance was determined by Student's *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

Human post-mortem brain tissues were obtained from the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry, King's College London.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from Alzheimer's Research UK, MRC and the Wellcome Trust. Funding to pay the Open Access publication charges for this article was provided by the Wellcome Trust.

REFERENCES

- De Vos, K.J., Grierson, A.J., Ackerley, S. and Miller, C.C.J. (2008) Role of axonal transport in neurodegenerative diseases. *Annu. Rev. Neurosci.*, **31**, 151–173.
- Chevalier-Larsen, E. and Holzbaur, E.L. (2006) Axonal transport and neurodegenerative disease. *Biochim. Biophys. Acta*, **1762**, 1094–1108.
- Morfini, G.A., Burns, M., Binder, L.I., Kanaan, N.M., LaPointe, N., Bosco, D.A., Brown, R.H. Jr., Brown, H., Tiwari, A., Hayward, L. *et al.* (2009) Axonal transport defects in neurodegenerative diseases. *J. Neurosci.*, **29**, 12776–12786.
- Millecamps, S. and Julien, J.P. (2013) Axonal transport deficits and neurodegenerative diseases. *Nat. Rev. Neurosci.*, **14**, 161–176.
- De Strooper, B., Vassar, R. and Golde, T. (2010) The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat. Rev. Neurol.*, **6**, 99–107.
- Randall, A.D., Witton, J., Booth, C., Hynes-Allen, A. and Brown, J.T. (2010) The functional neurophysiology of the amyloid precursor protein (APP) processing pathway. *Neuropharmacology*, **59**, 243–267.
- Walsh, D.M. and Selkoe, D.J. (2004) Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron*, **44**, 181–193.
- Stokin, G.B., Lillo, C., Falzone, T.L., Brusch, R.G., Rockenstein, E., Mount, S.L., Raman, R., Davies, P., Masliah, E., Williams, D.S. *et al.* (2005) Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease. *Science*, **307**, 1282–1288.
- Vagnoni, A., Perkinton, M.S., Gray, E.H., Francis, P.T., Noble, W. and Miller, C.C.J. (2012) Calsynenin-1 mediates axonal transport of the amyloid precursor protein and regulates Abeta production. *Hum. Mol. Genet.*, **21**, 2845–2854.
- Steuble, M., Diep, T.M., Schatzle, P., Ludwig, A., Tagaya, M., Kunz, B. and Sonderegger, P. (2012) Calsynenin-1 shelters APP from proteolytic processing during anterograde axonal transport. *Biol. Open*, **1**, 761–774.
- Decker, H., Lo, K.Y., Unger, S.M., Ferreira, S.T. and Silverman, M.A. (2010) Amyloid-beta peptide oligomers disrupt axonal transport through an NMDA receptor-dependent mechanism that is mediated by glycogen synthase kinase 3beta in primary cultured hippocampal neurons. *J. Neurosci.*, **30**, 9166–9171.
- Hiruma, H., Katakura, T., Takahashi, S., Ichikawa, T. and Kawakami, T. (2003) Glutamate and amyloid beta-protein rapidly inhibit fast axonal transport in cultured rat hippocampal neurons by different mechanisms. *J. Neurosci.*, **23**, 8967–8977.
- Kasa, P., Papp, H., Kovacs, I., Forgon, M., Penke, B. and Yamaguchi, H. (2000) Human amyloid-beta1–42 applied *in vivo* inhibits the fast axonal transport of proteins in the sciatic nerve of rat. *Neurosci. Lett.*, **278**, 117–119.
- Pigino, G., Morfini, G., Atagi, Y., Deshpande, A., Yu, C., Jungbauer, L., Ladu, M., Busciglio, J. and Brady, S. (2009) Disruption of fast axonal transport is a pathogenic mechanism for intraneuronal amyloid beta. *Proc. Natl Acad. Sci. USA*, **106**, 5907–5912.
- Rui, Y., Tiwari, P., Xie, Z. and Zheng, J.Q. (2006) Acute impairment of mitochondrial trafficking by beta-amyloid peptides in hippocampal neurons. *J. Neurosci.*, **26**, 10480–10487.
- Wang, X., Perry, G., Smith, M.A. and Zhu, X. (2010) Amyloid-beta-derived diffusible ligands cause impaired axonal transport of mitochondria in neurons. *Neurodegenerative Diseases*, **7**, 56–59.
- Zempel, H., Thies, E., Mandelkow, E. and Mandelkow, E.M. (2010) Abeta oligomers cause localized Ca²⁺ elevation, missorting of endogenous tau into dendrites, tau phosphorylation, and destruction of microtubules and spines. *J. Neurosci.*, **30**, 11938–11950.
- Kaether, C., Skehel, P. and Dotti, C.G. (2000) Axonal membrane proteins are transported in distinct carriers: a two-color video microscopy study in cultured hippocampal neurons. *Mol. Biol. Cell*, **11**, 1213–1224.
- Szodorai, A., Kuan, Y.H., Hunzelmann, S., Engel, U., Sakane, A., Sasaki, T., Takai, Y., Kirsch, J., Muller, U., Beyreuther, K. *et al.* (2009) APP Anterograde transport requires Rab3A GTPase activity for assembly of the transport vesicle. *J. Neurosci.*, **29**, 14534–14544.
- Brunholz, S., Sisodia, S., Lorenzo, A., Deyts, C., Kins, S. and Morfini, G. (2011) Axonal transport of APP and the spatial regulation of APP cleavage and function in neuronal cells. *Exp. Brain Res.*, **217**, 353–364.
- Hirokawa, N., Noda, Y., Tanaka, Y. and Niwa, S. (2009) Kinesin superfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.*, **10**, 682–696.
- Falzone, T.L., Stokin, G.B., Lillo, C., Rodrigues, E.M., Westerman, E.L., Williams, D.S. and Goldstein, L.S. (2009) Axonal stress kinase activation and tau misbehavior induced by kinesin-1 transport defects. *J. Neurosci.*, **29**, 5758–5767.
- Kamal, A., Stokin, G.B., Yang, Z.H., Xia, C.H. and Goldstein, L.S.B. (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron*, **28**, 449–459.
- Lazarov, O., Morfini, G.A., Lee, E.B., Farah, M.H., Szodorai, A., DeBoer, S.R., Koliatsos, V.E., Kins, S., Lee, V.M., Wong, P.C. *et al.* (2005) Axonal transport, amyloid precursor protein, kinesin-1, and the processing apparatus: revisited. *J. Neurosci.*, **25**, 2386–2395.
- Ludwig, A., Blume, J., Diep, T.M., Yuan, J., Mateos, J.M., Leuthausen, K., Steuble, M., Streit, P. and Sonderegger, P. (2009) Calsynenins mediate TGN exit of APP in a kinesin-1-dependent manner. *Traffic*, **10**, 572–589.

26. Konecna, A., Frischknecht, R., Kinter, J., Ludwig, A., Steuble, M., Meskenaite, V., Indermuhle, M., Engel, M., Cen, C., Mateos, J.M. *et al.* (2006) Calsyntenin-1 docks vesicular cargo to kinesin-1. *Mol. Biol. Cell*, **17**, 3651–3663.
27. Araki, Y., Kawano, T., Taru, H., Saito, Y., Wada, S., Miyamoto, K., Kobayashi, H., Ishikawa, H.O., Ohsugi, Y., Yamamoto, T. *et al.* (2007) The novel cargo Alcadin induces vesicle association of kinesin-1 motor components and activates axonal transport. *EMBO J.*, **26**, 1475–1486.
28. Dodding, M.P., Mitter, R., Humphries, A.C. and Way, M. (2011) A kinesin-1 binding motif in vaccinia virus that is widespread throughout the human genome. *EMBO J.*, **30**, 4523–4538.
29. Vagnoni, A., Rodriguez, L., Manser, C., De Vos, K.J. and Miller, C.C.J. (2011) Phosphorylation of kinesin light chain-1 at serine-460 modulates binding and trafficking of calsyntenin-1. *J. Cell Sci.*, **124**, 1032–1042.
30. Kawano, T., Araseki, M., Araki, Y., Kinjo, M., Yamamoto, T. and Suzuki, T. (2012) A small peptide sequence is sufficient for initiating Kinesin-1 activation through part of TPR region of KLC1. *Traffic*, **13**, 834–848.
31. Horiuchi, D., Collins, C.A., Bhat, P., Barkus, R.V., Diantonio, A. and Saxton, W.M. (2007) Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. *Curr. Biol.*, **17**, 1313–1317.
32. Muresan, Z. and Muresan, V. (2005) Coordinated transport of phosphorylated amyloid-beta precursor protein and c-Jun NH2-terminal kinase-interacting protein-1. *J. Cell Biol.*, **171**, 615–625.
33. Kuan, Y.H., Gruebl, T., Soba, P., Eggert, I., Back, S., Kirsch, J., Beyreuther, K. and Kins, S. (2006) PAT1a Modulates intracellular transport and processing of amyloid precursor protein (APP), APLP1, and APLP2. *J. Biol. Chem.*, **281**, 40114–40123.
34. Zheng, P., Eastman, J., Vande Pol, S. and Pimplikar, S.W. (1998) PAT1, A microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein. *Proc. Natl Acad. Sci. USA*, **95**, 14745–14750.
35. Matsuda, S., Matsuda, Y. and D'Adamio, L. (2003) Amyloid beta protein precursor (AbetaPP), but not AbetaPP like protein-2, is bridged to the kinesin light chain by the scaffold JNK-interacting protein 1. *J. Biol. Chem.*, **278**, 38601–38606.
36. Inomata, H., Nakamura, Y., Hayakawa, A., Takata, H., Suzuki, T., Miyazawa, K. and Kitamura, N. (2003) A scaffold protein JIP-1b enhances amyloid precursor protein phosphorylation by JNK and its association with kinesin light chain 1. *J. Biol. Chem.*, **278**, 22946–22955.
37. Verhey, K.J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B.J., Rapoport, T.A. and Margolis, B. (2001) Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. *J. Cell Biol.*, **152**, 959–970.
38. Horiuchi, D., Barkus, R.V., Pilling, A.D., Gassman, A. and Saxton, W.M. (2005) APLP1, A kinesin binding JIP-1/JNK scaffold protein, influences the axonal transport of both vesicles and mitochondria in *Drosophila*. *Curr. Biol.*, **15**, 2137–2141.
39. Matsuda, S., Yasukawa, T., Homma, Y., Ito, Y., Niihara, T., Hiraki, T., Hira, S., Ohno, S., Kita, Y., Kawasumi, M. *et al.* (2001) c-Jun N-terminal kinase (JNK)-interacting protein-1b/isolet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK. *J. Neurosci.*, **21**, 6597–6607.
40. Taru, H., Iijima, K., Hase, M., Kirino, Y., Yagi, Y. and Suzuki, T. (2002) Interaction of Alzheimer's beta-amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. *J. Biol. Chem.*, **277**, 20070–20078.
41. Taru, H., Kirino, Y. and Suzuki, T. (2002) Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. *J. Biol. Chem.*, **277**, 27567–27574.
42. Dajas-Bailador, F., Jones, E.V. and Whitmarsh, A.J. (2008) The JIP1 scaffold protein regulates axonal development in cortical neurons. *Curr. Biol.*, **18**, 221–226.
43. D'Ambrosio, C., Arena, S., Fulcoli, G., Scheinfeld, M.H., Zhou, D., D'Adamio, L. and Scaloni, A. (2006) Hyperphosphorylation of JNK-interacting protein 1, a protein associated with Alzheimer disease. *Mol. Cell. Proteomics*, **5**, 97–113.
44. Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., Andrieu, P.D., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J. *et al.* (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.*, **76**, 173–181.
45. Ackerley, S., Thornhill, P., Grierson, A.J., Brownlees, J., Anderton, B.H., Leigh, P.N., Shaw, C.E. and Miller, C.C.J. (2003) Neurofilament heavy chain side-arm phosphorylation regulates axonal transport of neurofilaments. *J. Cell Biol.*, **161**, 489–495.
46. Goldsburly, C., Mocanu, M.M., Thies, E., Kaether, C., Haass, C., Keller, P., Biernat, J., Mandelkow, E. and Mandelkow, E.M. (2006) Inhibition of APP trafficking by tau protein does not increase the generation of amyloid-beta peptides. *Traffic*, **7**, 873–888.
47. Rodrigues, E.M., Weissmiller, A.M. and Goldstein, L.S. (2012) Enhanced beta-secretase processing alters APP axonal transport and leads to axonal defects. *Hum. Mol. Genet.*, **21**, 4587–4601.
48. Mandelkow, E.M., Thies, E., Trinczek, B., Biernat, J. and Mandelkow, E. (2004) MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. *J. Cell Biol.*, **167**, 99–110.
49. Araki, Y., Tomita, S., Yamaguchi, H., Miyagi, N., Sumioka, A., Kirino, Y. and Suzuki, T. (2003) Novel cadherin-related membrane proteins, Alcadeins, enhance the X11-like protein-mediated stabilization of amyloid beta-protein precursor metabolism. *J. Biol. Chem.*, **278**, 49448–49458.
50. Hill, K., Li, Y., Bennett, M., McKay, M., Zhu, X., Shern, J., Torre, E., Lah, J.J., Levey, A.I. and Kahn, R.A. (2003) Munc18 interacting proteins: ADP-ribosylation factor-dependent coat proteins that regulate the traffic of beta-Alzheimer's precursor protein. *J. Biol. Chem.*, **278**, 36032–36040.
51. McLoughlin, D.M. and Miller, C.C.J. (1996) The intracellular cytoplasmic domain of the Alzheimer's disease amyloid precursor protein interacts with phosphotyrosine binding domain proteins in the yeast two-hybrid system. *FEBS Lett.*, **397**, 197–200.
52. Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y. and Suzuki, T. (1999) Interaction of a neuron-specific protein containing PDZ domains with Alzheimer's amyloid precursor protein. *J. Biol. Chem.*, **274**, 2243–2254.
53. Lee, J.H., Lau, K.F., Perkinson, M.S., Standen, C.L., Rogelj, B., Falinska, A., McLoughlin, D.M. and Miller, C.C. (2004) The neuronal adaptor protein X11beta reduces Abeta levels and amyloid plaque formation in the brains of transgenic mice. *J. Biol. Chem.*, **279**, 49099–49104.
54. Mitchell, J.C., Ariff, B.B., Yates, D.M., Lau, K.F., Perkinson, M.S., Rogelj, B., Stephenson, J.D., Miller, C.C. and McLoughlin, D.M. (2009) X11beta rescues memory and long-term potentiation deficits in Alzheimer's disease APPswe Tg2576 mice. *Hum. Mol. Genet.*, **18**, 4492–4500.
55. Sano, Y., Syuzo-Takabatake, A., Nakaya, T., Saito, Y., Tomita, S., Itohara, S. and Suzuki, T. (2006) Enhanced amyloidogenic metabolism of APP in the X11L-deficient mouse brain. *J. Biol. Chem.*, **281**, 37853–37860.
56. Ittner, L.M., Ke, Y.D. and Gotz, J. (2009) Phosphorylated tau interacts with c-JUN N-terminal kinase (JNK) interacting protein 1 (JIP1) in Alzheimer's disease. *J. Biol. Chem.*, **284**, 20909–20916.
57. Hata, S., Fujishige, S., Araki, Y., Taniguchi, M., Urakami, K., Peskind, E., Akatsu, H., Araseki, M., Yamamoto, K., Martins, R.N. *et al.* (2011) Alternative processing of gamma-secretase substrates in common forms of mild cognitive impairment and Alzheimer's disease: evidence for gamma-secretase dysfunction. *Ann. Neurol.*, **69**, 1026–1031.
58. Hata, S., Taniguchi, M., Piao, Y., Ikeuchi, T., Fagan, A.M., Holtzman, D.M., Bateman, R., Sohrabi, H.R., Martins, R.N., Gandy, S. *et al.* (2012) Multiple gamma-secretase product peptides are coordinately increased in concentration in the cerebrospinal fluid of a subpopulation of sporadic Alzheimer's disease subjects. *Mol. Neurodegener.*, **7**, 16.
59. De Vos, K.J., Chapman, A.L., Tennant, M.E., Manser, C., Tudor, E.L., Lau, K.F., Brownlees, J., Ackerley, S., Shaw, P.J., McLoughlin, D.M. *et al.* (2007) Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal transport to reduce axonal mitochondria content. *Hum. Mol. Genet.*, **16**, 2720–2728.
60. Cousins, S.L., Hoey, S.E., Stephenson, F.A. and Perkinson, M.S. (2009) Amyloid precursor protein 695 associates with assembled NR2A- and NR2B-containing NMDA receptors to result in the enhancement of their cell surface delivery. *J. Neurochem.*, **111**, 1501–1513.
61. Hao, C.Y., Perkinson, M.S., Chan, W.W., Chan, H.Y., Miller, C.C. and Lau, K.F. (2011) GULP1 is a novel APP-interacting protein that alters APP processing. *Biochem. J.*, **436**, 631–639.
62. Morotz, G.M., De Vos, K.J., Vagnoni, A., Ackerley, S., Shaw, C.E. and Miller, C.C.J. (2012) Amyotrophic lateral sclerosis-associated mutant VAPBP56S perturbs calcium homeostasis to disrupt axonal transport of mitochondria. *Hum. Mol. Genet.*, **21**, 1979–1988.